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Growth, chemical composition and resistance to *Streptococcus iniae* challenge of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of dietary inositol

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Abstract

A study was undertaken to determine the influence of the dietary levels of inositol on growth performance, feed utilization, immune response and resistance of Nile tilapia to *Streptococcus iniae* challenge. Six isonitrogenous (38% crude protein), isocaloric (3300 kcal DE/kg) purified diets supplemented with graded levels of inositol (0.0, 50, 100, 200, 400 or 800 mg/kg diet) were fed to juvenile tilapia averaging 5.8 ± 0.1 g (mean ± S.E.M.) in triplicate aquaria twice daily to apparent satiation for 10 weeks. Supplementation of dietary inositol had no affect on final weight gain, feed intake, feed efficiency ratio and survival. Also, no significant differences were observed in total cell, red and white blood cell counts, hematocrit and plasma hemoglobin content among treatments. Hepatosomatic index was not significantly ($P>0.05$) affected by dietary treatments. Fish fed the 800-mg inositol diet had significantly lower muscle lipid content than the groups fed diets containing 200 mg inositol/kg or lower, but these values did not differ from that of fish fed the 400-mg inositol diet. Liver lipid content significantly decreased when dietary inositol was increased to 100 mg/kg diet or higher. Plasma lysozyme activity and antibody titers to *S. iniae* were not affected by supplementation of dietary inositol. Cumulative mortality of fish 14-day post challenge with *S. iniae* was likewise not affected by the dietary inositol levels. The results of this study indicate that Nile tilapia juveniles did not require an exogenous source of inositol for normal growth, feed utilization and erythropoiesis. Supplementation of dietary inositol had no effect on improving the resistance of tilapia to *S. iniae*

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infection. However, supplementation of inositol to purified diets is required to prevent lipotropic effect of inositol deficiency.

Keywords: Nile tilapia; Inositol; Growth response; Feed utilization; Disease resistance

1. Introduction

Inositol (*myo*-inositol) is widely distributed in plants and animals mainly as a structural component of biological membranes in the phospholipid form. Inositol phospholipids and their metabolites are universal second messengers in the signal transduction pathway. Biochemical functions of phosphatidylinositol include the mediation of cellular responses to external stimuli, nerve transmission and the regulation of enzyme activity through specific interactions with various proteins (Chang et al., 2001).

Inositol can be synthesized by various animal tissues through a ring closure of glucose-6-phosphate, catalyzed by the enzyme L-*myo*-inositol-phosphate synthase. However, in rodents, the capacity for inositol biosynthesis in vivo appears to be limited since dietary inositol is needed to prevent the development of inositol deficiency (Hayashi et al., 1974; Kukiss and Mookerjea, 1978; Chu and Geyer, 1982, 1983). Dietary inositol deficiency induced alopecia, growth retardation and lipodystrophy characterized by an abnormal lipid accumulation in liver and/or small intestine accompanied by a hypolipoproteinemia, increased levels of cholesterol and triacylglycerols but decreased amount of total phospholipids, phosphotidylcholine, phosphotidylethanolamine and phosphotidylinositol (Chu and Hegsted, 1980; Chu and Geyer, 1981, 1983; Holub, 1986).

In fish, nutritional evidence suggests that metabolic synthesis of inositol occurs to some degree in liver, kidney and other tissues (Aoe and Masuda, 1967; Burtle and Lovell, 1988; Deng et al., 2002). For some fish species, however, de novo synthesis is inadequate to support their metabolic needs and, thus, require an exogenous source of this vitamin. Poor growth, anorexia, fin erosion, dark skin coloration, slow gastric emptying, and decreased cholinesterase and certain aminotransferase activities were observed in rainbow trout, chinook salmon, common carp, red sea bream, yellowtail and Japanese eel fed inositol-deficient diets (McLaren et al., 1947; Halver, 1953; Aoe and Masuda, 1967; Kitamura et al., 1967; Yone et al., 1971; Arai et al., 1972). The reported quantitative requirements of dietary inositol for these species range from 250 to 500 mg/kg diet (NRC, 1993). Other fish species such as channel catfish, Asian sea bass and sunshine bass do not require a dietary supplementation of inositol for normal growth and development (Burtle and Lovell, 1988; Boonyaratpalin and Wanakowat, 1993; Deng et al., 2002). Burtle and Lovell (1988) and Deng et al. (2002) demonstrated that de novo synthesis of inositol was sufficient for normal growth of channel catfish and sunshine bass. They suggested, however, that the synthesis of inositol by the microorganisms in the digestive tract was an unimportant source of this vitamin (Burtle and Lovell, 1988; Deng et al., 2002). To our knowledge, the essentiality of inositol in the diet of Nile tilapia has not yet been established.

The present study was conducted to evaluate the influence of the dietary levels of inositol on growth performance, feed utilization, chemical composition and resistance of juvenile Nile tilapia to *Streptococcus iniae* challenge.

2. Materials and methods

2.1. Experimental animals

Juvenile Nile tilapia (*Oreochromis niloticus*) produced at our laboratory were acclimated to the laboratory conditions and the basal experimental diet without inositol supplementation for 2 weeks. At the end of the acclimation period, fish with an average weight of 5.8 ± 0.1 g (mean $\pm$ S.E.M., $n = 60$) were randomly selected and stocked in eighteen 57-l aquaria at a density of 30 fish/aquarium. The aquaria were supplied with flow-though dechlorinated city water at an initial rate of 0.5 l/min and increased gradually to about 1.0 l/min by the 8th week of the trial. Water flow rates were checked and adjusted twice daily to ensure proper water exchange. Water temperature was maintained constant by a centralized heater at 26 ± 2 °C (mean $\pm$ S.E.M.). The water was continuously aerated with air stones and the photoperiod maintained on a 12:12-h light/dark schedule. Dissolved oxygen and temperature in three randomly chosen aquaria were measured four times a week using an YSI model 58 Oxygen Meter (Yellow Spring Instrument, Yellow Spring, OH). During the trial, water temperature averaged 26.4 ± 0.2 °C and dissolved oxygen averaged 5.7 ± 0.1 mg/ml.

2.2. Feed and feeding

A purified basal diet formulated to contain approximately 38% crude protein, 7% crude lipid and 3300 kcal of digestible energy (DE)/kg based on the feedstuff values reported in NRC (1993) (Table 1) was supplemented with *myo*-inositol (USB, Cleveland, OH) at 0, 50, 100, 200, 400 or 800 mg/kg diet at the expense of celufil. Dietary ingredients were thoroughly mixed in a Hobart mixer and passed through a 2.38-mm diameter die in a Hobart meat grinder. The pellets were air-dried at room temperature to a moisture content of about 10%, broken into small pieces and stored in a freezer at -8 °C until used. Each experimental diet was randomly assigned to triplicate aquaria. Fish were fed to apparent satiation twice daily (between 0700–0800 and 1500–1600 h) for 10 weeks. The quantity of feed consumed was recorded daily by calculating the differences in the weight of feed before the first feeding and after the last feeding. All the aquaria were cleaned thoroughly once every other week, between sampling, by scrubbing and siphoning accumulated wastes. On cleaning days, fish received only the afternoon meal.

2.3. Growth measurements

Fish in each aquarium were counted and group weighed every 2 weeks, following 16 h of feed deprivation. When fish were removed for weighing, aquaria were cleaned thoroughly and three-forth of the water drained. No feeding was done on sampling days.

Table 1
Composition of basal diet

Ingredient	Percent in diet
Casein, vitamin-free	35.50
Gelatin	11.20
Corn starch	34.30
Corn oil	4.00
Cod liver oil	3.00
Mineral premix[a]	4.00
Vitamin premix (inositol-free)[b]	1.00
Carboxymethyl cellulose	3.00
Ethoxyquin	0.02
Celufil	3.98

[a] Mineral premix: Williams and Briggs (1963) salt mixture supplemented in mg/kg diet with cobalt chloride, 4; aluminum potassium sulfate dodecahydrate, 5.18; and sodium selenite, 0.32.

[b] Vitamin premix: (mg/kg diet unless otherwise stated): vitamin A-acetate, 4000 IU; vitamin D_3, 2000 IU; vitamin K, 10; α-tocopheryl acetate, 50; thiamin, 10; riboflavin, 12; pyridoxine, 10; panthothenic acid, 32; nicotinic acid, 80; folic acid, 5; biotin, 0.2; cyanocobaltamine, 0.01; choline chloride, 400; L-ascorbyl acid-2-polyphosphate (15% vitamin C activity), 75.

2.4. Hepatosomatic index, and liver and muscle lipid content

At the end of the growth trial (10 weeks), four fish per aquarium were randomly chosen, anesthetized with tricaine methanesulfonate (MS-222) at 150 mg/ml and individually weighed. Livers were removed and weighed for determination of hepatosomatic index, and stored frozen at −80 °C for determination of total lipid content. Muscle samples were taken from the lateral dorsal part of the body, below the dorsal fin and above the lateral line, and frozen at −80 °C for measurement of total lipid content. Liver and muscle samples of fish from the same aquarium were pooled for determination of lipid content by the method of Folch et al. (1957).

2.5. Hematological and immunological assays

At the end of the growth trial, blood was sampled from the caudal vein of four anesthetized fish per aquarium for determination of hematocrit, hemoglobin, and total cell and red blood cell counts. Another group of four fish per aquarium were also bled. Blood was immediately centrifuged at $1000 \times g$ for 5 min and plasma stored frozen at −80 °C for subsequent assays for lysozyme activity and pre-challenge agglutinating antibody titer against *S. iniae*. Hematocrit, total blood cell count, red blood cell count and hemoglobin were determined as described by Barros et al. (2002). Plasma lysozyme activity was measured according to the method described by Parry et al. (1965).

2.6. Bacterial challenge

S. iniae (ARS 98-60) from an outbreak of streptococcal disease was used to challenge fish by intraperitoneal injection. Frozen stock-culture of *S. iniae* was grown in tryptic soy

broth (TSB, Difco Laboratories, Sparks, MD) for 24 h at 27 °C. The concentration of the culture was adjusted to an optical density of 1.2 at 540 nm, using a spectrophotometer, to give a *S. iniae* concentration of 1×10^8 colony-forming units/ml.

At the end of the growth trial, 15 fish from each aquarium were challenged by intraperitoneal injection with 100 μl of *S. iniae* solution containing 10^8 cells/ml. After injection, the fish were returned to their respective aquaria. Twenty-four hours after challenge, the flow rate was established (0.5–0.6 l/min) and each group of fish was fed twice daily with the same experimental diet that was assigned in the growth trial. Fish behavior and mortality were recorded twice a day for 14 days. At the end of the challenge trial (day 15), blood samples were collected from four surviving fish by venipuncture, centrifuged at $1000 \times g$ and plasma stored frozen at −80 °C for subsequent determination of post-challenge agglutinating antibody titer against *S. iniae*.

Agglutinating antibody titer against *S. iniae* (ARS 98-60) in pre- and post-challenge plasma was determined by modifying the method of Chen and Light (1994). *S. iniae* was grown for 24 h in TSB at 27 °C and killed in 1% formalin. The cells were centrifuged at $3000 \times g$ for 15 min. The resulting pellet was suspended in sterile PBS and washed three times. The bacterial concentration was adjusted to an optical density of 0.8 at 540 nm. Each well of a 96-round-bottomed microtiter plate was plated with 15 μl of sterile PBS and then 15 μl of plasma was added to the first well of each row and mixed. Twofold serial dilutions were then made by adding 15 μl of diluted plasma into the remaining wells. An equal volume (15 μl) of bacterial suspension was added to each well. Thus, the initial dilution of the plasma was 1:4. Positive plasma from a *S. iniae* infected fish and negative (PBS) were used as assay controls. The plates were covered with plastic film and incubated at room temperature for 16 h. The agglutination end point was established at the last dilution where cell agglutination was visible.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the general linear model of SAS (SAS Institute, 1993). Duncan's multiple-range test was used to determine

Table 2
Average weight gain, dry matter feed intake, feed efficiency ratio and survival of Nile tilapia fed diets containing various levels of inositol[a]

Inositol added (mg/kg diet)	Weight gain (g/fish)	Feed intake (g DM/fish)	FER[b]	Survival (%)
0	51.7	41.3	1.25	98.9
50	51.8	41.4	1.24	93.3
100	51.5	40.0	1.29	98.9
200	50.0	40.4	1.24	93.4
400	50.6	40.5	1.25	95.5
800	60.2	45.8	1.31	96.7
Pooled S.E.M.	4.3	2.4	0.03	1.9

[a] No significantly differences ($P > 0.05$) were observed among treatment means.
[b] Feed efficiency ratio: (wet weight gain/dry feed intake).

Table 3
Mean total cell count (TCC), red blood cell count (RBC), hematocrit, hemoglobin and plasma lysozyme of Nile tilapia fed diets containing various levels of inositol[a]

Inositol added (mg/kg diet)	TCC 10^6/μl	RBC 10^6/μl	Hematocrit (%)	Hemoglobin (g/dl)	Lysozyme (μg/ml)
0	3.26	2.75	30.9	9.40	42.0
50	3.31	2.70	31.9	9.96	28.4
100	3.44	2.78	33.9	9.70	36.7
200	3.76	3.02	31.5	10.62	31.9
400	3.34	2.88	31.5	9.37	30.7
800	3.21	2.69	30.7	9.87	27.4
Pooled S.E.M.	0.23	0.16	1.9	0.41	5.2

[a] No significantly differences ($P>0.05$) were observed among treatment means.

significant differences among individual treatment means. Differences were considered significant at a probability level of 0.05.

3. Results

Mean final weight gain, feed intake, feed efficiency ratio and survival are presented in Table 2. Dietary inositol supplementation did not significantly affect the total weight gain, feed intake or feed efficiency ratio. Mortality of the experimental fish was low and not correlated with the experimental diets. No clinical deficiency signs were observed in fish fed various dietary levels of inositol.

Total cell count, red blood cell count, hematocrit and hemoglobin were not significantly affected by dietary concentrations of inositol (Table 3). Plasma lysozyme activity was likewise not affected by dietary levels of inositol.

Hepatosomatic index was not significantly affected by the dietary treatment (Table 4). Muscle total lipid content was significantly lower in fish fed the diet with 800 mg/kg of inositol than those of the other groups. No significant differences were observed among the values for fish fed dietary inositol levels of 400 mg/kg or lower. Liver lipid content was

Table 4
Hepatosomatic index, liver and muscle lipid content and of Nile tilapia fed diets containing various levels of inositol[1]

Inositol added (mg/kg diet)	HSI[2] (%)	Liver lipid (% wet weight)	Muscle lipid (% wet weight)
0	1.71	16.0[a]	0.82[a]
50	1.69	13.5[a,b]	0.76[a]
100	1.66	10.7[b,c]	0.69[a]
200	1.50	9.4[c]	0.73[a]
400	1.91	9.1[c]	0.58[a,b]
800	1.65	8.9[c]	0.37[b]
Pooled S.E.M.	0.12	1.2	0.09

[1] Column means having the same superscript are not significantly different ($P>0.05$).
[2] Hepatosomatic index: (liver weight/body weight) × 100.

Table 5
Mean days of first death, cumulative mortality, agglutinating antibody titer against *S. iniae* of Nile tilapia at 14 days post-immersion challenge[a]

Inositol added (mg/kg diet)	Days to first mortality	Cumulative mortality (%)	Antibody titter (log_{10})
0.0	2.0	71.1	1.59
50	2.0	73.3	1.05
100	2.3	82.2	0.82
200	2.0	77.8	1.58
400	2.0	80.0	1.15
800	2.0	86.7	1.23
Pooled S.E.M.	0.14	6.1	0.31

[a] No significant differences ($P>0.05$) among treatment means.

highest in fish fed the inositol-unsupplemented diet, but this did not differ from that of fish fed the 50-mg inositol diet. Increasing dietary levels of inositol to 100 mg/kg or higher significantly decreased liver lipid content. However, there were no significant differences among the values for fish fed diets containing 50 and 100 mg inositol/kg diet.

The average number of days at which the first mortality occurred following *S. iniae* challenge and cumulative mortality of tilapia 14 days post-challenge with *S. iniae* were not significantly affected by the dietary levels of inositol (Table 5). Post-challenge antibody titers against *S. iniae* were likewise not affected by dietary inositol levels.

4. Discussion

Results of the present study indicate that weight gain, feed intake, feed efficiency and survival of Nile tilapia were not affected by dietary levels of inositol. No overt deficiency signs reported for other species were observed among fish fed various dietary levels of inositol. This agrees with previous results reported for channel catfish (Burtle and Lovell, 1988), Asian sea bass (Boonyaratpalin and Wanakowat, 1993) and sunshine bass (Deng et al., 2002). Metabolic synthesis of inositol detected in several organs such as liver and brain was sufficient to meet the requirements of channel catfish (Burtle and Lovell, 1988) and sunshine bass (Deng et al., 2002). These authors also demonstrated that the synthesis of inositol by intestinal microflora was an insignificant source of inositol. Although the rates of inositol synthesis in different tissues were not evaluated in our study, the lack of differences on growth performance and the absence of gross deficiency signs in fish fed diets devoid of inositol indicate that Nile tilapia, like channel catfish, Asian sea bass and sunshine bass, do not have a requirement for exogenous source of inositol for normal growth and development. This is in contrast to rainbow trout, chinook salmon, common carp, red sea bream, yellowtail and Japanese eel which have been shown to requirement exogenous source of dietary inositol for normal growth and to prevent of clinical deficiency signs (Mclaren et al., 1947; Halver, 1953; Aoe and Masuda, 1967; Kitamura et al., 1967; Yone et al., 1971; Arai et al., 1972).

The susceptibility of fish to low dietary levels of inositol appeared to vary with species, life stage, the rate of inositol biosynthesis, as well as physiological and nutritional stresses

(Kukiss and Mookerjea, 1978; NRC, 1993). Dietary source and level of carbohydrate and lipid influenced inositol requirement in some species (Kukiss and Mookerjea, 1978; Chu and Geyer, 1983). Burtle and Lovell (1988), however, observed that altering levels of carbohydrate (dextrin) and lipid (fish and soybean oils) in diets for channel catfish affected weight gain but did not influence the need for an exogenous source of *myo*-inositol.

Anemia has been reported as a clinical sign of inositol deficiency in salmonids (Halver, 1982). Waagbø et al. (1998) observed a positive correlation between blood hemoglobin concentrations and dietary levels of inositol in Atlantic salmon. Burtle and Lovell (1988) observed that omission of inositol from the diet, with or without the addition of an antibiotic, did not affect hematocrit of channel catfish. In our study, supplementation of dietary inositol had no effect on mean total cell count, red cell count, hematocrit and hemoglobin of Nile tilapia. Thus, Nile tilapia were probably able to synthesize sufficient quantity of inositol for normal erithropoiesis.

The role of inositol as a lipotropic factor for various animal species has been well documented (Holub, 1982, 1986; Holub et al., 1982). Inositol deficiency has been reported to be associated with a variety of lipid metabolic disturbances resulting in accumulation of lipids in liver, decreased hepatic lipoprotein output and intestinal lipodystrophy. This was due to interference with chylomicron assembly and secretion, and thus impairment of lymphatic transport of dietary fat (Hegsted et al., 1973; Chu and Geyer, 1983). High acetyl-CoA carboxylase activity as well as lipolysis in the adipose tissue may also contribute to the accumulation of triacylglycerols in the liver during inositol deficiency. When dietary inositol levels are reestablished, synthesis and turnover of phosphatidylinositol were increased, followed by enhanced lipid clearance, leading to liver and/or intestinal recovery (Hayashi et al., 1974; Chu and Geyer, 1983). In rainbow trout, Holub et al. (1982) observed increased neutral lipid content in the liver, elevated levels of cholesterol and triacylglycerols, but decreased amounts of total phospholipids in fish fed a diet devoid of inositol. In Atlantic salmon, plasma triacylglycerol was negatively correlated to dietary inositol supplementation but plasma cholesterol was unaffected (Waagbø et al., 1998). Thus, the significantly higher accumulation of lipid in liver and muscle of fish fed dietary inositol at levels less than 100 and 400 mg/kg, respectively, observed in our study, could be an indication of inositol deficiency. However, based on growth and hematological data, it appears that Nile tilapia can synthesize inositol to meet the requirement for growth and prevention of gross clinical deficiency signs but insufficient to prevent alteration of lipid metabolism. Liver and muscle lipid concentrations observed in this study, however, are within the normal range reported for fish (Burtle and Lovell, 1988; Waagbø et al., 1998; Tocher et al., 2002).

Streptococcal disease of tilapia caused by the bacterium *S. iniae* seriously affects the intensive farming of this species (Shoemaker and Klesius, 1997). Deficiencies or excesses of any dietary nutrients may have profound effects on disease development and survival of fish (Lim and Webster, 2001). However, no study has been conducted on the effect of dietary inositol on immune response and disease resistance in fish. Based on the biochemical functions of inositol, Holub (1982) suggested a possible role of inositol in the prevention and treatment of certain diseases. In the present study, serum lysozyme activity and antibody titer against *S. iniae* were not affected by supplementation of dietary inositol. Likewise, inositol supplementation had no influence on mortality of Nile tilapia

infected with *S. iniae*. This may be because tilapia were able to synthesize sufficient amount of inositol and did not require exogenous source of inositol to maintain normal immune function and resistance against *S. iniae* infection.

The results of this study indicate supplementation of dietary inositol had no effects on growth, feed utilization efficiency, hematological values, immune response and the resistance of Nile tilapia to *S. iniae* infection. However, supplementation of inositol to purified diets at levels of 100 and 400 mg/kg diet was required to lower total lipid content in liver and muscle, respectively. Thus, Nile tilapia can probably synthesize inositol in quantity sufficient to meet the requirement for normal growth, feed utilization, immune function and disease resistance but insufficient to prevent alteration of lipid metabolism. However, because inositol is widely distributed in common feed ingredients, practical diets should contain sufficient levels of this vitamin to meet various metabolic needs of Nile tilapia.

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